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Vaxfectin® enhances both antibody and *in vitro* T cell responses to each component of a 5-gene *Plasmodium falciparum* plasmid DNA vaccine mixture administered at low doses

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ABSTRACT

We previously reported the capacity of the cationic lipid-based formulation, Vaxfectin®, to enhance the immunogenicity and protective efficacy of a low dose plasmid DNA vaccine against *Plasmodium yoelii* malaria in mice. Here, we have extended this finding to human *Plasmodium falciparum* genes, evaluating the immune enhancing effect of Vaxfectin® formulation on a mixture, designated CSLAM, of five plasmid DNA vaccines encoding antigens from the sporozoite (*Pf*CSP, *Pf*SSP2/TRAP), intrahepatic (*Pf*LSA1), and erythrocytic (*Pf*AMA1, *Pf*MSP1) life cycle stages of *P. falciparum* administered at 2, 10 or 50 μg doses. Vaxfectin® formulation enhanced both antibody and cellular immune responses to each component of the multi-antigen vaccine mixture, as assessed by ELISA, IFAT, and IFN-γ ELIspot, respectively. There was no apparent antigenic competition, as indicated by comparison of responses induced in mice immunized with *Pf*CSP vs. CSLAM. These data showing that Vaxfectin® can enhance the immunogenicity of plasmid DNA vaccines administered at low doses per body weight, and in combinations, has important clinical implications for the development of a vaccine against malaria, as well as against other public health threats.

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1. Introduction

In humans and animals, immunization with *Plasmodium* sporozoites attenuated by radiation, such that their development is halted at the liver stage, can protect against subsequent sporozoite challenge. Protection is believed to be mediated by cell-mediated immune responses directed against multiple proteins expressed by irradiated sporozoites after entry and limited development in hepatocytes [1,2]. Naturally acquired clinical immunity, observed in residents of malaria endemic areas who have experienced repeated malaria infections, is effective at preventing severe malaria; and is thought to be mediated by antibodies directed against multiple antigens expressed in the blood stage [3,4]. These models establish the feasibility of developing a malaria vaccine but, despite extensive research spanning decades, an efficacious malaria vaccine is not yet available [5–7]. The level of protection obtained to date with subunit vaccines based on a single antigen has been suboptimal [8–13], and there is increasing recognition of the need for vaccines targeting more than one antigen and more than one stage of the parasite life cycle, and that induce both cellular and humoral immunity. Accordingly, the research focus has moved towards development of a multi-stage multi-immune response vaccine comprising antigens expressed in the liver stage and targeted by T-cell responses as well as antigens expressed in the blood-stage and targeted by antibody responses [14]. By reducing the numbers of parasites emerging from the liver (T-cell immune responses directed against liver stage

Abbreviations: APCs, antigen presenting cells; CI, confidence interval; D-D, DNA–DNA immunization regimen; D-V, DNA-recombinant viral boost immunization regimen; ELISA, enzyme-linked immunosorbent assay; ELISpot, enzyme-linked immunospot; IFA, immunofluorescent antibody test; IFN-γ, interferon-gamma; pDNA, plasmid DNA; *Pf*, *Plasmodium falciparum*; CSP, circumsporozoite protein; SSP2, sporozoite surface protein 2; LSA1, liver stage antigen-1; AMA1, apical merozoite antigen-1; $MSP1_{42}$ (3D7 strain), merozoite surface protein-1; SFC, spot-forming cells.

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14. ABSTRACT

We previously reported the capacity of the cationic lipid-based formulation. Vaxfec tin"'. to enhance the immunogcniciry and protective efficacy of a low dose plasmid DNA vaccine against Plasmodium yoelii malaria in mice. Here. we have extended this finding to human Plasmodiumfalciparum genes. evalua ting the immune en hancing effect ofVaxfectin"' formulation on a mixture. designated CSLAM. of five plasmid DNA vaccines encoding antigens from the sporozoi te (Pj(SP. Pj5SP2{fRAP). intrahepatic (PjlSA 1). and erythrocytic (1'/AMA 1. I~SP1) life cycle stages of 1'. falcipurum administered at 2. I 0 or 50 1-1-g doses. Vaxfec tin"' formulation enhanced both antibody and ce ll ular immune responses ro each component of the multi-an tigen vaccine mixture. as assessed by ELISA, IFAT, and IFN-"(ELl spot. respectively. There was no apparent antigenic competition. as indicated by comparison of responses induced in mice immunized with P}t:SP vs. CSLAM. These data showing that Vaxfectin "' can enhance the immunogeniciry of plasmid DNA vaccines administered at low doses per body weigh t, and in combinations. has important clin ical implications for the development of a vaccine against malaria. as well as against other public health threats.

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antigens) and priming the immune system to antigens that will be boosted by infection from natural exposure (antibody responses directed against blood stage antigens), it is hypothesized that one will reduce the severity and mortality due to *Plasmodium falciparum* malaria. Other research is being directed towards the development of whole organism vaccines such as the irradiated sporozoite vaccine being commercialized by Sanaria Inc. [15] and genetically attenuated parasites [16–19] which rely on the expression of the entire repertoire of parasite antigens expressed in given stage of the parasite life cycle. It is well established that antigen variability is a major obstacle to malaria vaccine development [20,21]. The immunological challenges to developing effective malaria interventions have been recently reviewed [22].

Based on the two models of human immunity against malaria – irradiated sporozoite immunization and naturally acquired immunity – we have sought to develop a DNA-based malaria vaccine which relies upon the induction of antibody and T-cell responses against multiple parasite proteins expressed in different stages of the parasite life cycle. Our previous studies of mice immunized with a mixture of plasmid DNA (pDNA) encoding nine *P. falciparum* (3D7 strain) antigens revealed suppressive effects in the multi-antigen mixture which could be associated with a specific subset of the nine antigens [23]. Those data led us to down-select a core panel of five antigens, designated CSLAM (CSP, SSP2, LSA1, AMA1, and MSP1), for further development and evaluation. The pre-erythrocytic stage antigens (CSP, SSP2, and LSA1) are designed to induce T-cell responses against liver stage antigens, and the erythrocytic stage antigens (AMA1 and MSP1) are aimed at inducing responses directed against blood stage parasites. Since AMA1 and MSP1 are also expressed in sporozoites and liver stage parasites [24–26] they are expected to also contribute to hepatic stage immunity. The underlying rationale is that, upon vaccination, the combination of responses from the pre-erythrocytic and erythrocytic stage components could result in sterile protection by eliminating all or most of the parasites developing in the liver (pre-erythrocytic immunity) together with a backup immunity that would limit and clear any breakthrough parasites that evaded the liver stage immunity to develop blood stage infections (erythrocytic immunity).

One limitation of multi-antigen vaccines is the amount of pDNA injected and the potential need for a highly concentrated pDNA mixture which could affect the efficiency of transcription and translation and thereby result in a suppressed or suboptimal immune response against target antigens. Moreover, the modest immunogenicity of some pDNA vaccines to date in human and nonhuman primate studies that involved needle injections may be attributed, at least in part, to the relatively low dose per body weight as compared to murine studies. The upper limit of total pDNA that can be injected is influenced by both the concentration and viscosity of the vaccine and the injection volume. Finally, the manufacturing costs associated with high dose and requirements to include multiple antigens of interest for some pathogens, detract from the often-cited advantages of pDNA vaccines.

In both animal and human studies that involved needle injections, the immune responses induced by DNA prime followed by DNA boost (D-D) immunization regimens often follow a dose response [27,28]. This implies that in order to induce optimal responses against all antigenic components in an optimal pDNA mixture without significant interference from some specific plasmids, one may still have to meet a certain dose threshold for all individual pDNA components in the mixture. To avoid possible suppressive effects due to the use of high dose pDNA and to reduce cost, we embarked on a series of studies which capitalized on the ability to formulate low dose pDNA with Vaxfectin® for induction of robust antibody and T-cell responses. We have previously shown that, compared to PBS-formulated pDNA, low dose *Py*CSP pDNA formulated in Vaxfectin® enhanced levels of antibodies, IFN-γ responses, and sterile protection after challenge [29]. In other studies in mice, rabbits, and other animals, the use of Vaxfectin® formulation to deliver vaccine antigens for anthrax and influenza induced strong systemic, long-lived, and antigen-specific antibody responses [30–36,38,39]. Vaxfectin®-formulated influenza H5 hemagglutinin-containing pDNA vaccines have undergone Phase I testing in humans with encouraging results [37]. Vaxfectin® was also shown to enhance antibody and T-cell responses to a protein-based influenza vaccine in mice [40].

In the current study, we hypothesized that, when tested at the same pDNA dose, the breadth of the total anti-malarial T-cell response induced to a 5-gene vaccine, CSLAM, will exceed the response induced to a single gene vaccine. Our second hypothesis was that a homologous D-D immunization regimen with low dose pDNA, involving single antigen or with a 5-gene vaccine formulated with Vaxfectin® will yield immune responses comparable to those obtained with higher dose pDNA of the same vaccines given without formulation. In general, priming with pDNA followed by boosting with recombinant vaccinia virus (D-V) dramatically increases the immune response as compared to responses induced by D-D regimens [41,42]. Therefore, our third hypothesis was that a D-D immunization regimen with low dose pDNA involving single antigen or with a 5-gene vaccine formulated with Vaxfectin® will yield enhanced immune responses comparable to or approaching those obtained with unformulated low dose pDNA administered in a heterologous DNA prime-viral boost (D-V) immunization regimen.

2. Materials and methods

2.1. Mice

Six to 8-week-old female inbred BALB/cByJ (H-2^d) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and used for cellular response studies. Six to 8-week-old female outbred CD-1 mice obtained from Charles River Laboratories (Wilmington, MA) were used for antibody response studies.

2.2. Plasmids

Plasmid DNA (pDNA) stocks encoding each of five *P. falciparum* antigens (3D7 strain), CSP, SSP2, LSA1, AMA1, and MSP1, as well as empty vector without insert, VR1020, were produced by PureSyn, Inc. (Malvern, PA). All plasmids have been previously described [23]. All pDNA stocks were ≥90% supercoiled. Vaxfectin®-formulated pDNA stock endotoxin levels were less than 30 EU/mg, while the unformulated pDNA stock endotoxin levels were less than 7.5 EU/mg.

2.3. P. falciparum recombinant virus (NYVAC-Pf7)

The recombinant attenuated vaccinia virus, NYVAC-*Pf*7, used in boosting experiments has been previously described [23,43,44]. NYVAC-*Pf*7 expresses seven *P. falciparum* antigens comprising *Pf* CSP, *Pf* SSP2, *Pf* LSA1, *Pf* SERA, *Pf* MSP1, *Pf* AMA1, and *Pf* Pfs25. The *Pf* CSP, *Pf* SSP2, *Pf* AMA1, and *Pf* Pfs25 antigens derive from the NF54/3D7 clone of *P. falciparum*, the *Pf* LSA1 antigen derives from the NF54 strain of *P. falciparum*, and *Pf*MSP1 and SERA derive from the Uganda-Palo Alto and FCR3 strains, respectively.

2.4. Vaxfectin® formulations

Plasmid/Vaxfectin® formulations were prepared as previously described [29]. Briefly, Vaxfectin® in sterile water for injection

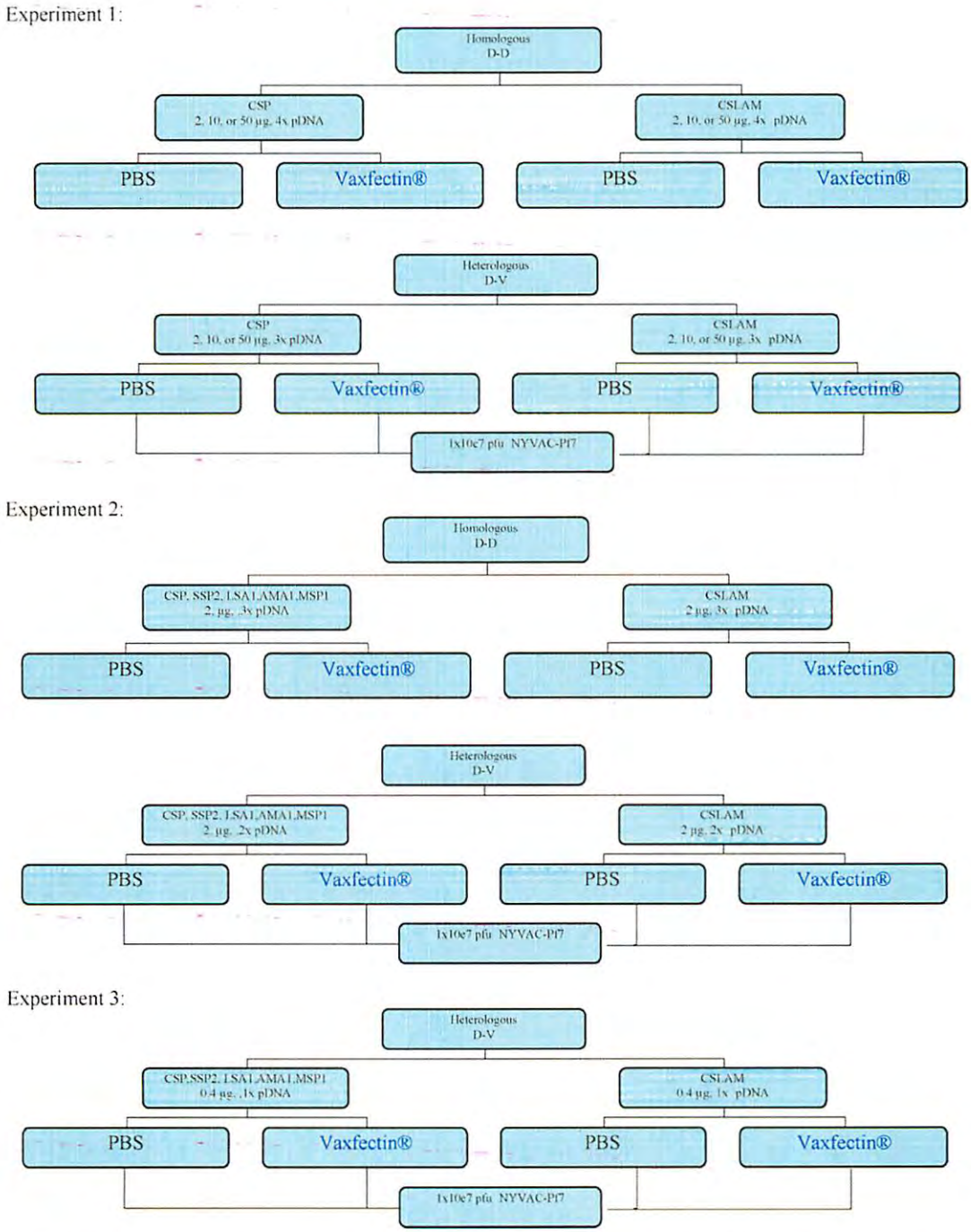

Fig. 1. Fluxogram of immunizations. All injections were given at 4-week intervals and samples for antibody and/or IFN-γ ELISpot responses were assayed at 2 weeks after last injection.

(SWFI) was added to an equal volume of pDNA (single plasmid or plasmid mixture) at twice the desired final concentration in 2× PBS (20 mM sodium phosphate, pH 7.2, 1.8% NaCl). Formulations were mixed by gentle inversion. The final molar ratio of pDNA:cationic lipid was 4:1.

2.5. Immunizations

Mice ($n = 14$ per group for antibody studies in CD-1 mice and $n = 8$ per group for T-cell studies in BALB/c mice) received intramuscular injections in the tibialis anterior muscle pDNA formulated in PBS or Vaxfectin® using insulin syringes (0.3 ml; Becton Dickinson 309301) with 29G1/2 needles. Individual plasmids or the CSLAM combination were administered at the same total pDNA dose and in the same number of sites; therefore, for each antigen, the actual amount of plasmid in the 5-gene-CSLAM plasmid mixture was always one-fifth as compared to the individual plasmid vaccine.

In Experiment 1, three pDNA doses that included 2, 10 and 50 μg of CSP and CSLAM pDNA vaccines in PBS or formulated in Vaxfectin® were tested in either homologous D-D or heterologous D-V immunization regimens (D-D and D-V immunized in parallel). The homologous immunization regimen groups received 4 doses of 2, 10 or 50 μg pDNA in PBS or formulated in Vaxfectin®, whereas

the heterologous immunization groups received 3 doses of 2, 10 or 50 μg pDNA in PBS or Vaxfectin® followed by an intramuscular boost with 1 x 10^7 pfu NYVAC-*Pf*7 recombinant virus. Three control groups included mice injected 3 times with empty vector pDNA formulated in PBS or Vaxfectin® and boosted with NYVAC-*Pf*7, and naive mice. Injections were carried out at 4-week intervals and samples for antibody and IFN-γ ELISpot responses were assayed at 2 weeks after the last injection.

In Experiment 2, each of the five plasmids included in the CSLAM vaccine was injected individually at a dose of 2 μg per mouse. CSLAM vaccine was also injected at a dose of 2 μg per mouse. After 2 priming injections with pDNA, the homologous immunization regimen groups received a third pDNA immunization, while the heterologous immunization groups received an intramuscular boost with 1 × 10^7 pfu NYVAC-*Pf*7 recombinant virus. IFN-γ ELISpot responses were assessed in pooled splenocytes from each group 2 weeks after the last injection.

In Experiment 3, each of the five plasmids included in the CSLAM vaccine was tested at an even lower dose of 0.4 μg per mouse. After a single pDNA priming, all primed animals received an intramuscular boost with 1 × 10^7 pfu NYVAC-*Pf*7 recombinant virus. Another group of mice received no pDNA priming, but an intramuscular injection with 1 × 10^7 pfu NYVAC-*Pf*7 recombinant virus. IFN-γ ELISpot responses were assessed in individual mouse splenocytes 2 weeks after the last injection.

A fluxogram of all three experiments is illustrated in Fig. 1.

2.6. Antibody assays

Individual sera obtained 2 weeks after the last injection (Experiment 1) were analyzed for reactivity with *P. falciparum* sporozoites and blood stage parasites by IFA as previously described [8]. Sera were also analyzed by standard ELISA as previously described [45]. The expression, purification, and characterization of the five *P. falciparum* recombinant proteins used as capture antigens, CSP, SSP2, LSA1, AMA1, and MSP1 has been previously reported [23].

2.7. IFN-γ ELISpot assays

Detection of antigen-specific IFN-γ producing cells was performed as previously described [23] with modification. Since H-2^d restricted T cell epitopes have not been defined for all antigens studied here, and because responses could be directed at multiple epitopes on a given antigen, we used transfected A20 cells (ATCC) as antigen presenting cells (APCs) in the ELISpot assay. For the 5 antigens, this approach offered an efficient and more practical means of measuring T-cell responses in all experimental groups simultaneously against all five antigenic targets prepared in the same manner. Due to the large number of groups of mice involved, pooled splenocytes were used in some experiments (Experiments 1 and 2). For the pooled cell assays, assessments were done at least twice using previously frozen cells. The data presented herein for Experiment 1 utilized frozen splenocytes, and Experiments 2 and 3 utilized fresh splenocytes for the ELISpot assays. We had previously compared the results of fresh or frozen cells assays and found that the magnitude and pattern of responses were similar (Sedegah, unpublished).

Seven APCs were prepared by transfecting A20 cells with plasmid encoding CSP, SSP2, LSA1, AMA1, MSP1, CSLAM mixture, or unmodified plasmid VR1020, using the AMAXA nucleofector system (AMAXA Biosystems/Lonza Colgone AG, Basel, Switzerland) according to manufacturer's instructions. Eighteen to 24 h after transfection, transfected cells were washed three times with complete medium, suspended in 2 ml complete medium, irradiated at 10K rads, counted, and used as APCs in the ELISpot assay [23]. Splenocytes were stimulated *in vitro* with different A20-transfected APCs and the number of *Pf* antigen-specific IFN-γ-secreting spot-forming cells was evaluated after a 36-h culture period. Splenocytes were tested at 400K, 200K, and 100K per well while APCs cells were tested at 100K per well. Assays were done in triplicate and the number of IFN-γ-secreting cells, recognized as spot-forming cells (SFC) was counted using an automated ELISpot Reader manufactured by Autolmmun Diagnostika (AID) (GmbH, Strassberg, Germany). The number of antigen-specific IFN-γ SFC in test wells was corrected for background by subtracting the mean number of SFC of the negative control wells (A20 cells transfected with empty plasmid VR1020) and the mean of the test sample was calculated. Data were presented as the number of IFN-γ-secreting SFC per million spleen cells.

2.8. Statistical analysis

IFA and ELISA titers generated from Experiment 1 were log transformed for analysis, and all calculations of confidence intervals were carried out with log transformed data. The mean of the log of the titer in each group and the difference in the mean log titer between corresponding Vaxfectin® and PBS groups are reported. Positive log titer differences where the 95% confidence interval (CI) does not involve a 0 or a negative number represent a significant difference. Statistical analysis of the antibody data was performed with Stata/SE version 8.0.

For the IFN-γ ELISpot data analysis in Experiment 3, data generated from individual mice (8 mice/group) immunized with the D-V regimen were analyzed in a 2-way analysis of variance, conducted with log transformed or untransformed data for three data sets, namely (1) the individual vaccines tested against the individ-

Table 1
IFA response in CD-1 mice to *P. falciparum* vaccines given by D-D and D-V regimens (Experiment 1).

Regimen	Dose (μg)	CSP vaccine			CSLAM vaccine					
		Sporozoite IFA			Sporozoite IFA			Blood Stage IFA		
		VAX	PBS	Difference log titer (95% CI)	VAX	PBS	Difference log titer (95% CI)	VAX	PBS	Difference log titer (95% CI)
D-D	2	5,653	2,826	0.3 (0.03, 0.57)	545	552	0 (−0.55, 0.56)	4,064	1452	0.45 (−0.06, 0.96)
	10	9,275	7,994	0.06 (−0.15, 0.27)	2,436	1,159	0.32 (0.09, 0.55)	1,689	2560	−0.18 (−0.80, 0.44)
	50	16,800	4,873	0.54 (0.24, 0.83)	2,970	4,363	−0.17 (−0.41, 0.08)	1,140	3121	−0.44 (−1.12, 0.25)
D-V	2	17,653	9,275	0.28 (−0.09, 0.64)	11,880	3,121	0.58 (0.20, 0.96)	5,120	2970	0.24 (−0.26, 0.73)
	10	33,601	12,058	0.44 (0.11, 0.78)	21,519	7,994	0.43 (0.16, 0.70)	10,240	4413	0.37 (−0.02, 0.75)
	50	19,491	24,965	−0.11 (−0.39, 0.17)	15,217	13,116	0.06 (−0.23, 0.36)	2,970	8400	−0.45 (−1.12, 0.22)

Sera from individual mice ($n = 14$) were assayed and geometric mean endpoint titers for each group against *P. falciparum* sporozoites or blood stages by IFA are reported. To compare the effect of Vaxfectin® formulation on antibody response, the individual endpoint antibody titers were log transformed and the difference in the mean log titers between corresponding Vaxfectin® and PBS groups and the 95% confidence interval (CI) around the difference were obtained and are reported in the "Difference log titer (95% CI)" column. Positive log titer differences where the CI does not involve a negative number or 0 represent significant difference (highlighted).

ual A20 APCs, (2) the individual vaccines tested against A20-CSLAM APCs, and (3) the CSLAM vaccine against individual and A20-CSLAM APCs. Pairwise comparisons of the group means were made with the Tukey–Kramer multiple comparison procedure at the (0.05) level of significance. Statistical analysis was performed using the NCSS software (NCSS, 2007).

3. Results

3.1. Effect of Vaxfectin® formulation on parasite-specific or antigen-specific antibody responses induced by immunization with CSLAM and CSP vaccines

Outbred CD-1 mice ($n = 14$/group) were immunized with 2, 10 or 50 μg doses of CSP or CSLAM pDNA vaccines in PBS or Vaxfectin® via homologous D-D or heterologous D-V regimens, as described in Section 2. Sera collected 2 weeks after the last immunization were assayed for parasite-specific antibodies by IFA and for antigen-specific antibodies by ELISA. IFA antibody titers to sporozoites were higher in mice immunized with CSP pDNA alone than in mice that received the CSLAM pDNA mixture (Table 1). However, this did not reflect significant suppressive effects due to the multi-antigen combination since comparable antibody levels were attained when the actual amount of CSP in the CSLAM was taken into account (2 and 10 μg CSP equivalent to 10 and 50 μg CSLAM respectively). As expected, mice immunized with CSLAM had a wide breadth of response as indicated by detectable IFA responses against blood stages as well as sporozoites (Table 1) and by ELISA against each of the CSLAM proteins (Table 2). Formulation of CSLAM and CSP vaccines with Vaxfectin® generally led to an increase in both IFA and ELISA titers, reaching statistical significance in some cases (Tables 1 and 2). The immune enhancement conferred by Vaxfectin® formulation was generally more pronounced at the lowest dose tested (2 μg) and in suboptimal immunization regimens such as low dose pDNA and homologous D-D rather than heterologous D-V regimens (Tables 1 and 2), consistent with our previous findings in the *Plasmodium yoelii* CSP model [29]. Next, we evaluated whether formulating low dose pDNA with Vaxfectin® could result in antibody levels comparable to those achieved by higher pDNA doses without formulation. Data showed that this was generally the case since responses induced by 2 μg CSP pDNA formulated in Vaxfectin® were similar to those induced by 50 μg doses of CSP pDNA in PBS (Tables 1 and 2). In the case of CSLAM however, ELISA antibody responses induced against two of the 5 antigens, AMA1 and MSP1 by Vaxfectin® were not increased except in the case of 2 μg CSLAM pDNA formulated in Vaxfectin® in the D-V regimen (Table 2). We also asked whether Vaxfectin® formulation of pDNA would allow the homologous D-D regimen to achieve levels of antibody response approaching those achievable with the heterologous D-V regimen. Our results showed that while Vaxfectin® formulation enhanced antibody levels at each of the three doses tested, levels did not surpass those obtained by D-V immunization (Tables 1 and 2).

3.2. Use of multi-antigen antigen presenting cells (APCs) to measure P. falciparum antigen-specific IFN-γ ELISpot responses

We carried out a preliminary assay to evaluate the use of APCs expressing the 5 antigens simultaneously in IFN-γ ELISpot assay. Splenocytes from mice immunized with low dose pDNA (2 μg, D-D regimen) involving the 5 antigens injected as individuals (2 μg total dose) or as a mixture (CSLAM 2 μg total dose; 0.4 μg/plasmid) were stimulated *in vitro* with different APCs that included A20 cells transfected with plasmid encoding CSP, SSP2, LSA1, AMA1, MSP1, or CSLAM mixture.

Table 2
ELISA response in CD-1 mice to *P. falciparum* vaccines given by D-D and D-V regimens (Experiment 1).

Regimen	Dose (μg)	CSP vaccine			CSLAM vaccine																		
		CSP			CSP			SSP2			LSA1			AMA1			MSP						
		VAX	PBS	Difference log titer (95% CI)	VAX	PBS	Difference log titer (95% CI)	VAX	PBS	Difference log titer (95% CI)	VAX	PBS	Difference log titer (95% CI)	VAX	PBS	Difference log titer (95% CI)	VAX	PBS	Difference log titer (95% CI)	VAX	PBS	Difference log titer (95% CI)
D-D	2	7,372	1,868	0.6 (0.16, 1.03)	462	259	0.06 (−0.91, 1.04)	5	3	0.3 (0.05, 0.54)	8	7	0.01 (−0.46, 0.49)	1,893	3,486	−0.27 (−1.33, 0.80)	559	1144	−0.31 (−1.84, 1.22)			
	10	21,571	11,726	0.26 (−0.10, 0.63)	4,214	1,403	0.48 (0.03, 0.92)	31	6	0.72 (0.14, 1.57)	8	6	0.08 (−0.44, 0.60)	767	12,998	−1.22 (−2.25, −0.20)	2136	5267	−0.39 (−1.65, 0.87)			
	50	60,593	9,946	0.78 (0.15, 1.42)	9,873	5,197	0.28 (−0.28, 0.84)	50	36	0.14 (−0.76, 1.03)	14	15	−0.03 (−0.60, 0.54)	3,793	12,695	−0.52 (−1.37, 0.32)	934	7392	−0.9 (−2.08, 0.28)			
D-V	2	44,863	19,869	0.35 (−0.16, 0.87)	11,142	1,863	0.78 (0.07, 1.48)	81	62	0.12 (−0.38, 0.61)	76	21	0.56 (0.26, 0.86)	45,521	11,762	0.59 (−0.08, 1.25)	4889	876	0.75 (−0.85, 2.34)			
	10	62,921	13,168	0.68 (0.22, 1.14)	12,330	12,601	−0.01 (−0.53, 0.51)	305	199	0.18 (−0.53, 0.90)	117	69	0.23 (−0.32, 0.78)	39,076	74,768	−0.28 (−0.64, 0.08)	3520	4276	−0.08 (−1.34, 1.17)			
	50	50,805	83,813	−0.22 (−0.66, 0.23)	16,581	15,174	0.04 (−0.44, 0.52)	1154	821	0.15 (−.50, 0.80)	209	185	0.05 (−0.54, 0.65)	35,293	65,070	−0.27 (−0.72, 0.19)	3187	6023	−0.28 (−1.54, 0.99)			

Sera from individual mice ($n = 14$) were assayed and geometric mean endpoint titers for each group against *P. falciparum* recombinant proteins by ELISA are reported. To compare the effect of Vaxfectin® formulation on ELISA response, the individual endpoint antibody titers were log transformed and the difference in the mean log titers between corresponding Vaxfectin® and PBS groups and the 95% confidence interval (CI) around the difference were obtained and are reported in the "Difference log titer (95% CI)" column. Positive log titer differences where the CI does not involve a negative number or a 0 represent significant difference (highlighted).

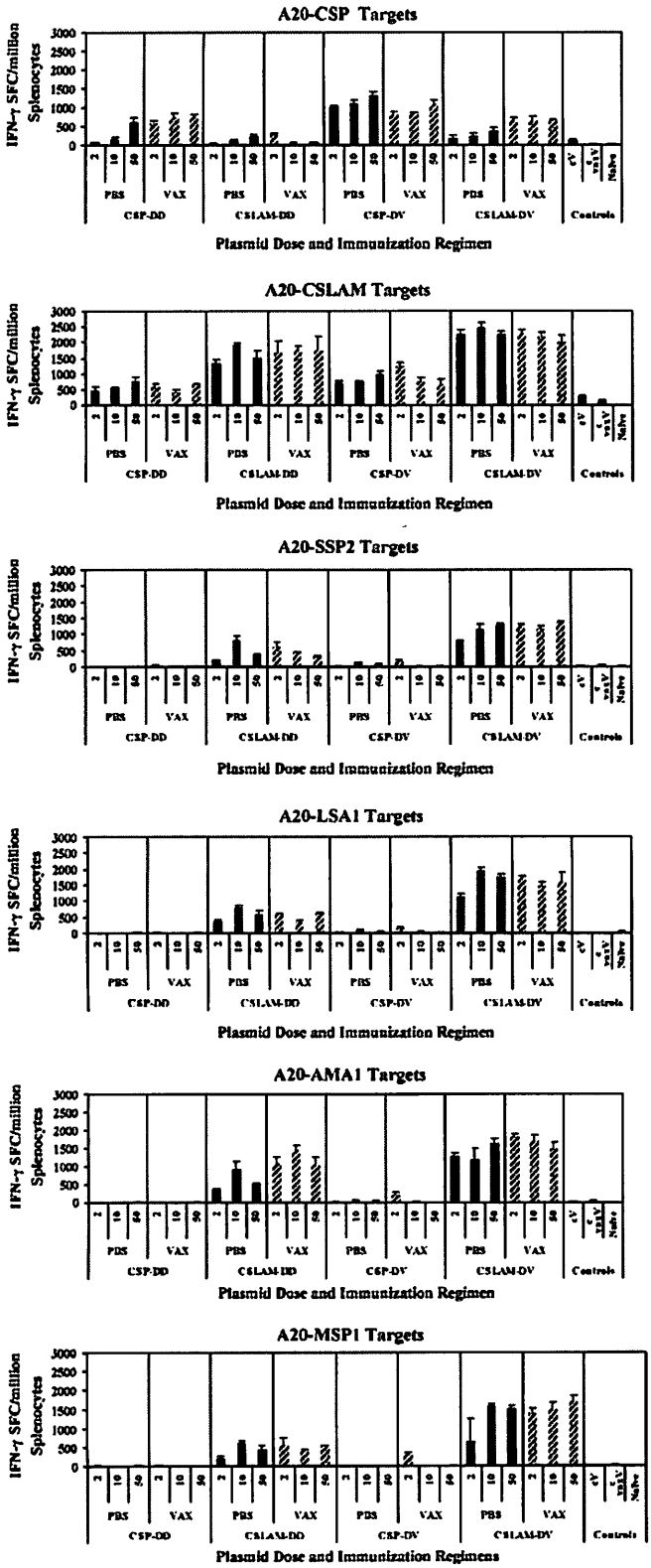

Fig. 2. IFN-γ ELISPOT response in BALB/c to CSP and CSLAM vaccines in D-D and D-V regimens (Experiment 1). IFN-γ ELISpot assays were carried out as described in Section 2. Freshly isolated spleen cells were pooled from 8 BALB/c mice per group 2 weeks after the last immunization and incubated with A20 cells transfected with *P. falciparum* CSP, SSP2, LSA1, AMA1, MSP1, CSLAM, or empty plasmid as control. Data is presented as antigen-specific IFN-γ spot-forming cells per million spleen cells (SFC) after background control responses have been subtracted. The *X*-axis reflects the immunization regimen: 2, 10, and 50 μg total pDNA/dose administration formulated in either PBS or Vaxfectin (Vax). Regimens were either the univalent pDNA vaccine PfCSP or the multivalent pDNA mixture CSLAM DNA alone regimen given as 4 homologous DNA doses 4 weeks apart (DD) or a prime-boost regimen given as 3 homologous DNA doses followed by a NYVAC-Pf7 boost dose each 4 weeks apart (DV). Control groups were given the DV regimen (3 doses empty pDNA, then 1 NYVAC-Pf7 boost) with pDNA in PBS (cV), or with pDNA in Vaxfectin (c vaxV). A third control group was naïve. Error bars reflect standard deviation of quadruplicate samples.

In an example of an assay that utilized 100K APCs, CSLAM immunized splenocytes stimulated with CSP, SSP2, LSA1, AMA1, MSP1, and CSLAM APCs yielded 5, 85, 88, 117, 154, and 316 SFC/million respectively; CSP immunized splenocytes stimulated with CSP and CSLAM APCs yielded 14 and 20 SFC/million, respectively; SSP2 immunized splenocytes stimulated with SSP2 or CSLAM APCs yielded 124, and 153 SFC/million, respectively; LSA1 immunized splenocytes stimulated with LSA1 or CSLAM APCs yielded 41 and 90 SFC/million, respectively; AMA1 immunized splenocytes stimulated with AMA1 or CSLAM APCs yielded 38 and 78 SFC/million respectively; and MSP1 immunized splenocytes stimulated with MSP1 or CSLAM APCs yielded 18 and 16 SFC/million, respectively. Overall, our general finding from these preliminary assays was that, while the responses detected against A20-CSLAM APCs were generally variable and higher than those detected against single antigen, the use of these APCs that expressed all 5 antigens served as a helpful additional tool in the evaluation of the total T-cell response induced to the 5-gene vaccine.

3.3. *Induction of P. falciparum antigen-specific IFN-γ ELISpot responses by immunization with CSP and CSLAM vaccines*

Splenocytes from BALB/c mice immunized with either CSP or CSLAM pDNA by D-D or D-V immunization regimens were assayed for IFN-γ responses against individual *Pf* antigen APCs. Seven different APCs were assayed, prepared by transfecting A20 cells with plasmid encoding CSP, SSP2, LSA1, AMA1, MSP1, CSLAM, or unmodified plasmid VR1020.

We first compared the magnitude of the CSP-specific IFN-γ response in mice immunized with 2, 10, and 50 μg of either CSP or CSLAM vaccine. Accordingly, CSP or CSLAM splenocytes were each incubated with A20-CSP APCs (A20 cells expressing only CSP) or A20-CSLAM APCs (A20 cells simultaneously expressing CSP, SSP2, LSA1, AMA1, and MSP1). Robust CSP-specific IFN-γ responses were detected by both CSP and CSLAM vaccines when A20-CSP APCs were used (Tables 3a and 3b, and Fig. 2). Secondly, each of the vaccines induced the strongest response against the matching APCs (Tables 3a and 3b, and Fig. 2). In general, it was noted that responses measured against A20-CSLAM APCs were generally higher regardless of vaccine (Tables 3a and 3b, and Fig. 2).

Next, we summed the IFN-γ response against all five antigenic APCs, as a measure of the total response induced by the multivalent CSLAM vaccine. Accordingly, splenocytes from CSP or CSLAM immunized mice were incubated with the panel of individual antigenic APCs comprising A20-CSP, A20-SSP2, A20-LSA1, A20-AMA1, and A20-MSP1. For each vaccine, we then summed the responses against all five APCs. Data with effectors from the CSLAM low dose group (2 μg) containing only one-fifth of the dose (0.4 μg) of the univalent CSP 2 μg dose group, confirmed our earlier finding that there was no evidence of suppression in the multi-antigen mixture (Tables 3a and 3b; 279 SFC using 0.4 μg CSP in CSLAM, compared to 582 SFC using 2 μg CSP). Similar findings were made when A20-CSLAM targets were used (Tables 3a and 3b; 1679 SFC using 0.4 μg CSP in CSLAM, compared to 598 SFC using 2 μg CSP).

For mice immunized with CSP via the D-D regimen, the summed response for the five antigen-expressing APCs was similar to the response obtained with A20-CSP APCs alone. For example, in the case of Vaxfectin® formulated CSP at 2, 10, and 50 μg doses, results

Table 3a
IFN-γ ELISpot response in BALB/c mice to *P. falciparum* CSP vaccine given by D-D and D-V regimens (Experiment 1).

Regimen	Dose (μg)	A20-CSP			A20-CSLAM		
		VAX	PBS	Ratio (VAX/PBS)	VAX	PBS	Ratio (VAX/PBS)
D-D	2	582	73	8	598	474	1.26
	10	750	153	4.89	390	548	0.71
	50	757	603	1.26	658	734	0.90
D-V	2	803	1026	0.78	1230	711	1.73
	10	806	1098	0.73	728	730	1.00
	50	1078	1317	0.82	642	1003	0.64

IFNγ ELISpot assays were carried out as described in Section 2. Freshly isolated spleen cells were pooled from 8 BALB/c mice per group 2 weeks after the last immunization and incubated with A20 cells transfected with *P. falciparum* CSP, CSLAM, or empty plasmid as control. Data is presented as antigen-specific IFN-γ spot-forming cells per million spleen cells (SFC) after background control responses have been subtracted. The ratio of SFC of corresponding Vaxfectin® and PBS groups were obtained and are reported in the "Ratio (VAX/PBS)" column.

obtained for A20-CSP vs. (A20-CSP + A20-SSP2 + A20-LSA1 + A20-AMA1 + A20-MSP1) were 582 vs. 602; 750 vs. 755; and 757 vs. 775 respectively.

In the Vaxfectin® formulated CSP at 2 μg dose group given via the D-V regimen, we obtained robust responses against CSP due to the CSP component from the NYVAC-*Pf* boost. However, expected background responses against the other four CSLAM antigens were also noted due to the immunogenicity of the NYVAC-*Pf7* boost even in the absence of prior antigen-specific priming with the non-CSP antigens. Summed responses induced by NYVAC-*Pf7* when injected alone against all 5 antigens was 95, which comprised of A20-CSP (19) + A20-SSP2 (44) + A20-LSA1 (0) + A20-AMA1 (21) + A20-MSP1 (11).

In other studies, we have reported observed suppressive effects with a 9-gene mixture which included the CSLAM antigens [23]. The CSLAM mixture was specifically down-selected from that 9-gene mixture on the basis of improved compatibility with negligible antigen interference [23].

3.4. Effect of Vaxfectin® formulation with low dose pDNA on antigen-specific IFN-γ responses in D-D and D-V immunization regimens

We have previously shown that Vaxfectin® formulation was most effective in enhancing immune responses at low doses of pDNA [29]. We hypothesized that immune responses obtained with low dose pDNA formulated with Vaxfectin® would be comparable to responses induced by higher doses of unformulated pDNA. Initial studies with 2, 10, and 50 μg pDNA in PBS, administered via a D-D regimen, showed a general dose response (Tables 3a and 3b, and Fig. 2). As hypothesized, Vaxfectin® formulation of low dose (2 μg) pDNA CSP and CSLAM vaccines administered via a D-D regimen resulted in high levels of response against A20-CSP and A20-CSLAM APCs that were comparable to or better than those achieved by unformulated high dose (10 and 50 μg) pDNA (Tables 3a and 3b, and Fig. 2). A similar trend was noted for the D-V immunization regimen, although the responses were more variable.

Our third hypothesis was that a D-D immunization regimen with low dose pDNA formulated with Vaxfectin® would yield enhanced immune responses comparable to those obtained with unformulated low dose pDNA administered in a heterologous DNA prime/viral boost (D-V) immunization regimen. Data showed that D-D immunization of Vaxfectin® formulated low dose (2 μg) pDNA CSLAM vaccine induced high responses against A20-CSP APCs, which were comparable to the responses induced by D-V immunization of unformulated low dose (2 μg) pDNA CSLAM vaccine (Tables 3a and 3b). However, for the non-CSP APCs, Vaxfectin® formulation of low dose (2 μg) pDNA enhanced D-D induced immune responses; however, levels approached but did not reach those obtained by D-V immunization with 2 μg unformulated pDNA CSLAM (Tables 3a and 3b, and Fig. 2).

3.5. IFN-γ ELISpot response to P. falciparum antigens by multivalent CSLAM and 5 individual antigens

Data presented above showed that the immune enhancing effect of Vaxfectin® formulation was generally greatest at low dose pDNA. Therefore, we next assessed the ability of Vaxfectin® formulation to enhance responses induced by low dose (2 μg) immunization with each of the five CSLAM plasmids injected individually or as the CSLAM mixture in Experiment 2; those studies used only two priming doses of pDNA instead of the three doses given in previous experiments in order to better dissect any potential differences (Table 4). In almost all cases, Vaxfectin® formulation enhanced responses against the respective APCs (Table 4).

Results from the experiments reported above suggested that the enhancing effects of Vaxfectin® formulation were more pronounced under suboptimal immunization conditions (namely, low pDNA dose and fewer primes). Since heterologous D-V immunization regimens are more immunogenic than homologous D-D regimens, we next asked whether Vaxfectin® formulation could enhance a suboptimal D-V regimen involving a single prime with an extremely low dose pDNA in Experiment 3. For those studies, mice were primed once with 0.4 μg pDNA encoding CSP, SSP2, LSA1, AMA, MSP1, or CSLAM, and boosted 4 weeks later with NYVAC-*Pf7*. Results showed that mice were sufficiently primed by the Vaxfectin® formulated low dose pDNA for boosting by NYVAC-*Pf7* in this abbreviated immunization regimen. Enhanced responses after priming with individual antigens were detected against all tested APCs, reaching statistical significance with almost all APCs (Table 5). Furthermore, as seen in the earlier experiments, no evidence of suppression in the mixture was noted taking into consideration that the amount of CSP pDNA was one-fifth the dose of the univalent vaccine being tested. Another group of mice that received the NYVAC-*Pf7* boost but no pDNA prime showed minimal responses (data not shown).

4. Discussion

Because of the complexity of the *Plasmodium* parasite life cycle, many believe that an effective subunit malaria vaccine will need to contain antigenic components from more than one developmental stage. Multi-antigen vaccines against malaria given as plasmids or in the form of recombinant mastocytoma-transfected cells have successfully protected mice [9,46] and monkeys [47,48] against malaria, establishing the feasibility of a multivalent malaria vaccine. Ease of combining pDNA vaccines, as compared to other conventional methods such as recombinant proteins and viral vectors, has made DNA an attractive platform for the delivery of

Table 3b
IFN-γ ELISpot response in BALB/c mice to *P. falciparum* CSLAM vaccine given by D-D and D-V regimens (Experiment 1).

Regimen	Dose (μg)	A20-CSP			A20-SSP2			A20-LSA1			A20-AMA1			A20-MSP1			A20-CSLAM			Summed CSLAM		
		VAX	PBS	Ratio (VAX/PBS)	VAX	PBS	Ratio (VAX/PBS)	VAX	PBS	Ratio (VAX/PBS)	VAX	PBS	Ratio (VAX/PBS)	VAX	PBS	Ratio (VAX/PBS)	VAX	PBS	Ratio (VAX/PBS)	VAX	PBS	Ratio (VAX/PBS)
D-D	2	279	51	5	628	214	3	573	374	2	1069	356	3	584	211	3	1679	1336	1	3133	1206	3
	10	53	143	0.37	455	818	0.56	327	788	0.41	1447	919	1.57	383	621	0.62	1768	1924	0.92	2665	3288	0.81
	50	51	244	0.21	308	373	0.83	598	591	1.01	1049	531	1.98	541	454	1.19	1724	1526	1.13	2546	2193	1.16
D-V	2	647	192	3.37	1265	807	1.57	1713	1133	1.51	1808	1277	1.42	1392	650	2.14	2278	2273	1.00	6825	4058	1.68
	10	653	250	2.61	1160	1155	1.00	1480	1963	0.75	1707	1188	1.44	1495	1593	0.94	2168	2468	0.88	6495	6150	1.06
	50	638	356	1.79	1368	1278	1.07	1584	1734	0.91	1476	1624	0.91	1686	1524	1.11	2016	2243	0.90	6751	6516	1.04

IFN-γ ELISpot assays were carried out as described in Section 2. Freshly isolated spleen cells were pooled from 8 BALB/c mice per group 2 weeks after the last immunization and incubated with A20 cells transfected with *P. falciparum* CSP, SSP2, LSA1, AMA1, MSP1, CSLAM, or empty plasmid as control. Data is presented as antigen-specific IFN-γ spot-forming cells per million spleen cells (SFC) after background control responses have been subtracted. The ratio of SFC of corresponding Vaxfectin® and PBS groups were obtained and are reported in the "Ratio (VAX/PBS)" column.

Table 4
IFN-γ ELISpot response in BALB/c mice to *P. falciparum* individual and CSLAM vaccines given by D-D and D-V regimens (Experiment 2).

Regimen	Vaccine	Individual and CSLAM vaccines vs. individual A20 targets															Summed CSLAM response		
		A20-CSP			A20-SSP2			A20-LSA1			A20-AMA1			A20-MSP1					
		VAX	PBS	Ratio (VAX/PBS)	VAX	PBS	Ratio (VAX/PBS)	VAX	PBS	Ratio (VAX/PBS)	VAX	PBS	Ratio (VAX/PBS)	VAX	PBS	Ratio (VAX/PBS)	VAX	PBS	Ratio (VAX/PBS)
D-D	Individual	490	157	3.1	2293	522	4.4	1007	783	1.3	1828	1183	1.5	840	353	2.4			
	CSLAM	220	57	3.9	620	460	1.3	597	447	1.3	1223	1160	1.1	1617	1037	1.6	4,277	3160	1.4
D-V	Individual	1663	377	4.4	3970	3840	1.0	4473	3680	1.2	4067	2373	1.7	2540	1600	1.6			
	CSLAM	270	292	0.9	1237	888	1.4	2823	1312	2.2	2860	1598	1.8	3897	2088	1.9	11,087	6178	1.8

Regimen	Individual and CSLAM vaccines vs. A20-CSLAM targets																	
	CSP			SSP2			LSA1			AMA1			MSP1			CSLAM		
	VAX	PBS	Ratio (VAX/PBS)	VAX	PBS	Ratio (VAX/PBS)	VAX	PBS	Ratio (VAX/PBS)	VAX	PBS	Ratio (VAX/PBS)	VAX	PBS	Ratio (VAX/PBS)	VAX	PBS	Ratio (VAX/PBS)
D-D	517	163	3.2	2.433	458	5.3	1080	740	1.5	1402	783	1.8	643	147	4.4	2433	2140	1.1
D-V	2240	683	3.3	3823	3747	1.0	4447	4177	1.1	3993	2163	1.8	2597	1940	1.3	4663	3368	1.4

IFN-γ ELISpot assays were carried out as described in Section 2. Freshly isolated spleen cells were pooled from 8 BALB/c mice per group 2 weeks after the last immunization (individual antigens or CSLAM), and incubated with A20 cells transfected with *P. falciparum* CSP, SSP2, LSA1, AMA1, MSP1, CSLAM, or empty plasmid as control. Data is presented as antigen-specific IFN-γ spot-forming cells per million spleen cells (SFC) after background control responses have been subtracted. The ratio of SFC of corresponding Vaxfectin® and PBS groups were obtained and are reported in the "Ratio (VAX/PBS)" column.

Table 5
IFN-γ ELISpot response in BALB/c mice to *P. falciparum* individual and CSLAM vaccines given by low dose (0.4 μg pDNA) D-V regimen only (Experiment 3).

Vaccine	Individual and CSLAM vaccines vs. individual A20 targets																	
	A20-CSP			A20-SSP2			A20-LSA1			A20-AMA1			A20-MSP1			Summed CSLAM response		
	VAX	PBS	Ratio (VAX/PBS)	VAX	PBS	Ratio (VAX/PBS)	VAX	PBS	Ratio (VAX/PBS)	VAX	PBS	Ratio (VAX/PBS)	VAX	PBS	Ratio (VAX/PBS)	VAX	PBS	Ratio (VAX/PBS)
Individual	185	22	8.6 $P=0.002$	1284	768	1.7 $P=0.0047$	1151	339	3.4 $P=0.0024$	572	301	1.9 $P=0.0078$	250	68	3.7 $P=0.16$			
CSLAM	125	99	1.3 $P=0.47$	524	273	1.9 $P=0.008$	599	309	1.9 $P=0.079$	257	144	1.8 $P=0.028$	480	528	0.9 $P=0.78$	1986	1353	1.5

Individual and CSLAM vaccines vs. A20-CSLAM targets

CSP vaccine			SSP2 vaccine			LSA1 vaccine			AMA1 vaccine			MSP1 vaccine			CSLAM vaccine		
VAX	PBS	Ratio (VAX/PBS)	VAX	PBS	Ratio (VAX/PBS)	VAX	PBS	Ratio (VAX/PBS)	VAX	PBS	Ratio (VAX/PBS)	VAX	PBS	Ratio (VAX/PBS)	VAX	PBS	Ratio (VAX/PBS)
581	173	3.4 $P<0.0001$	1223	636	1.9 $P=0.0003$	1185	498	2.4 $P=0.013$	934	596	1.6 $P=0.047$	457	197	2.3 $P=0.049$	1305	1113	1.2 $P=0.39$

IFN-γ ELISpot assays were carried out as described in Section 2. Freshly isolated spleen cells from individual BALB/c mice were evaluated ($n=8$) 2 weeks after the last immunization (individual antigens or CSLAM), and incubated with A20 cells transfected with *P. falciparum* CSP, SSP2, LSA1, AMA1, MSP1, CSLAM, or empty plasmid as control. Data is presented as antigen-specific IFN-γ spot-forming cells per million spleen cells (SFC) after background control responses have been subtracted. The ratio of SFC of corresponding Vaxfectin® and PBS groups were obtained and are reported in the "Ratio (VAX/PBS)" column. Results of Ratio of VAX/PBS comparisons that reached level of statistical significance (described in Section 2.8) are shown by *P* values.

malaria vaccines. Our previous data indicated that heterologous prime-boost immunization regimens, such as priming with pDNA and boosting with recombinant virus, are more efficacious than homologous immunization regimens [41,49]. The complex logistics and costs associated with manufacturing a second vaccine platform could be avoided if a suitable means of enhancing the immunogenicity of pDNA vaccines could be identified.

We have previously reported reduced immune responses to the antigenic components of a 5- and 9-gene mixture as compared to the responses obtained when given as individual plasmids [23,50]. Subsequent plasmid elimination studies led us to identify a 5-gene mixture, designated CSLAM, that was capable of inducing multi-target responses without serious suppressive effects. Studies with mixtures of this down-selected CSLAM mixture in nonhuman primates have shown no adverse effects due to antigen combination [51].

Herein, we have used this 5-gene mixture to address the effect of dose and plasmid formulation for optimal multi-antigen immunogenicity. We show that, with homologous DNA (D-D) immunization regimens, formulation of low dose plasmid with Vaxfectin® enhances immune responses, inducing levels comparable to those obtained with higher dose pDNA without formulation. We further show a general trend whereby the immune responses induced by D-D immunization regimens with a low dose pDNA formulation with Vaxfectin® was higher compared with unformulated pDNA, levels did not reach those induced with unformulated pDNA in a heterologous DNA prime-recombinant viral boost (D-V) regimen. This is a significant finding since the use of a homologous vaccine platform is more cost effective and easier to manufacture and administer than a heterologous multiple platform vaccine. This was not the first demonstration that Vaxfectin did not facilitate transfection [30]. An earlier study has suggested that the enhanced antibody responses are IL-6 dependent [40].

It should be noted that the readout of the current studies was immunogenicity (both T cell and antibody responses) since protection induced by *P. falciparum* constructs cannot be evaluated in preclinical models. However, the results reported here are consistent with data previously generated in the *P. yoelii* model which showed that Vaxfectin® formulation of low dose *Py*CSP plasmid DNA vaccines enhanced T cell and antibody responses as well as protective efficacy against *Plasmodium* sporozoite challenge in mice, as compared to PBS-formulated pDNA [29].

Our data further validate our previous down-selection of the 5-gene CSLAM vaccine [23,50]. Overall, our data establish that the multivalent vaccine CSLAM vaccine can induce robust and broad antibody and T cell responses against each component of the mixture in the apparent absence of antigen interference. The apparent absence of antigenic competition with plasmid DNA mixtures noted here, for both antibody and cellular responses, is consistent with data generated in Aotus monkeys where no significant difference in antigen-specific ELISA titers was noted in monkeys immunized with a mixture of *P. falciparum* DNA vaccines encoding apical merozoite antigen-1 (AMA1), erythrocyte binding protein-175 (EBA-175) and merozoite surface protein-1 (MSP1) as compared to monkeys immunized with each of the individual vaccines [52]. Other studies in mice have demonstrated that a mixture of two plasmid DNA vaccines can confer synergistic or additive effects on protection against sporozoite challenge [53]. The data presented here provide additional experimental evidence in support of the concept of multivalent vaccination. While the superiority of this multi-target, multi-immune response approach will have to await clinical testing with challenge, it is expected that multivalent vaccines, especially those targeted against more than one stage in the parasite life cycle, would be more protective than univalent vaccines.

Herein, we also report a novel approach to evaluation of a multivalent vaccine, whereby A20 transfectants expressing either single antigens or all five antigenic APCs simultaneously (A20-CSLAM) were used as APCs for the *in vitro* T cell assays. This made it possible for us to estimate the total response against the multivalent CSLAM vaccine by two methods, namely (1) summing responses induced against each of the five individual antigenic A20-APCs, or (2) assaying responses against a single target expressing all five antigens simultaneously. We found that, though the sum of responses against each of the five individual antigenic APCs was generally higher than the response against the CSLAM APCs, the pattern of responses was similar. This method is likely to be a useful complement to other studies of multivalent vaccines.

In other studies in the *P. yoelii* model, we have evaluated another cationic lipid formulation, DMRIE-DOPE. Our data showed that DMRIE-DOPE enhanced antibody response at all pDNA doses tested (0.4–50 μg) but reduced both IFN-γ responses and protective efficacy against sporozoite challenge, as compared with unformulated pDNA (Sedegah, unpublished). In contrast, our data with Vaxfectin® reported here show that Vaxfectin® preferentially enhanced both cell-mediated immunity and humoral immunity, and surpassed the level of antibody responses induced by DMRI-DOPE formulation (Sedegah, unpublished). Others have evaluated cationic distearoyl phosphatidylcholine (DSPC) liposomes in Leishmania vaccine studies and report long-term immunity in mice when the adjuvant was added with the immunodominant 63-kDa glycoprotein (gp63) of *Leishmania donovani* [54]. Chemical adjuvants for plasmid DNA vaccines which include liposomes, polymers and microparticles have been investigated extensively and liposomes and polymer adjuvants have proved effective in some models but not others [reviewed in 55].

In the current studies, Vaxfectin® formulation enhanced immune responses with fewer priming doses and reduced concentrations of pDNA which are advantageous for vaccine development in general, and for the development of a vaccine against complex pathogens such as malaria in particular. Preliminary human safety and immunogenicity data from a Phase I trial to evaluate a monovalent and trivalent Vaxfectin®-formulated H5N1 pandemic influenza DNA vaccines in healthy volunteers suggests that pDNA vaccines can achieve potentially protective levels of antibody responses in humans [37]. The aim is to use Vaxfectin® formulation to optimize pDNA priming of immune responses that could potentially eliminate the need for boosting with the viral vector vaccine. The finding of a well-tolerated safety profile and no vaccine-related serious adverse events in humans after immunization with Vaxfectin®-formulated pDNA [37] support further development of Vaxfectin®-formulated multivalent malaria pDNA vaccines for humans either as part of a prime-boost regimen that involves a recombinant virus, such as NYVAC-*Pf7* (which has already been evaluated in humans and showed to induce partial protection) [44], or as a stand alone DNA-DNA vaccine.

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